UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 37: 134–140, 2018 *Original paper*
Originalni naučni rad

ALTERATIONS IN ARGINASE-NO-SYNTHASE SYSTEM OF SPERMATOZOA IN HUMAN SUBJECTS WITH DIFFERENT FERTILITY POTENTIAL

PROMENE U SISTEMU ARGINAZA-NO-SINTAZE U SPERAMATOZOMIMA MUŠKARACA SA RAZLIČITIM FERTILITENTNIM POTENCIJALOM

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Background: Infertility is an important worldwide problem which affects 10–15% of couples globally. Altered NO production has also been implicated in the pathogenesis of the male infertility. The present study was designed to evaluate the changes in the activity of NO-synthase (NOS) and arginase in spermatozoa of patients with infertility.
Methods: The total NOS, Ca^{2+}-dependent constitutive (cNOS) and Ca^{2+}-independent inducible (iNOS) activity and arginase activity were assessed in sperm cells of patients with different forms of pathospermia.
Results: We found a significant increase in iNOS activity, but significantly decreased cNOS and arginase activity in sperm cells of infertile men vs fertile, normozoospermic men ($p<0.001$). The arginase/NOS ratio significantly decreased compared to control group. The iNOS/cNOS ratio was drastically increased in patients with decreased fertility potential indicating predominance of iNOS. Men with leukocytospermia were distinguished to have the most express iNOS activity.
Conclusions: These observations provide evidence for a disturbed balance between the L-arginine metabolic pathways in sperm cells of infertile men. This imbalance includes the considerable activation of the inducible isoform of NO-synthase accompanied by significant inhibition of its constitutive isoform which indicates disturbances in NO production. In patients with decreased fertility potential the arginase/NOS was shifted towards predominance of iNOS-derived NO production.

Keywords: NO-synthase; arginase; male infertility; pathospermia

Uvod: Neplodnost je značajan svetski problem koji pogađa 10–15% parova globalno posmatrano. Izmenjeno stvaranje NO je verovatno uključeno u patogenezu muške neplodnosti. Ova istraživanja se bave procenom izmenjene aktivnosti NO-sintaze (NOS) i arginaze u spermatozomima neplodnih muškaraca.
Metode: Ukupni NOS, Ca^{2+}-zavisne (cNOS) i Ca^{2+}-nezavisno podukovane (iNOS) aktivnosti i aktivnost arginaze su procenjivane i ćelijama sperme kod pacijenata sa različitim oblicima patospermija.
Rezultati: Nađeno je značajno povećanje aktivnosti iNOS i značajnio smanjenje cNOS i aktivnosti arginaze u ćelijama sperme infertilnih muškaraca vs. fertilnih, normozospermnih muškaraca ($p < 0,001$). Odnos arginaza/NOS bio je značajno smanjen u odnosu na kontrolnu grupu. Odnos iNOS/cNOS je bio značajno povećan kod pacijenata sa smanjenim fertilitentnim potenicijalom ukazujući na predominantnost iNOS. Muškarci sa leukocitospermijom imali su mnogo izraženiju aktivnost iNOS.
Zaključak: Dobijeni rezultati ukazuju na raspodelu ravnoteže između metaboličkih puteva L-arginina u ćelijama sperme kod neplodnih muškaraca. Ova neravnoteža uključuje značajnu aktivnost izoformi NO-sintaze praćenom značajnom inhibicijom njenih konstitutivnih izoformi što ukazuje na distribuciju u produkciji NO. Kod pacijenata sa smanjenim fertilitentim potencijalom arginaza/NOS se pomera predominantno prema iNOS-zavisnoj produkciji NO.

Ključne reči: NO-sintaza, arginaza, muška neplodnost, patospermija

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Introduction

Infertility is an important worldwide socio-demographic and medical-biological problem for the most developed countries. It affects 10–15% of couples globally and approximately up to 40–50 % of infertility is caused by male factor (1). Defective functions of spermatozoa are the most common cause of male infertility (2). Although the biological bases of defective sperm functions are poorly understood, a number of factors, such as chromosome abnormalities, oxidative stress, infections, endocrinopathies, environmental factors, stress vulnerability and others, have been related to the pathophysiology of male infertility. Inflammation in the reproductive tract of men resulting in leukocytospermia has also been implicated as a cause of defective sperm function.

The precise biochemical mechanisms underlying the male infertility are not clearly understood. Altered NO production has also been implicated in the pathogenesis of the male infertility (3). NO is a messenger molecule functioning in variety of physiological functions in different cell types and tissues including in sperm cells. It is derived from L-arginine by NO synthases (NOS). Constitutive isoforms of NOS (cNOS) provide a NO production in physiological conditions, whereas inducible NOS (iNOS) is inactive in physiological conditions and is activated in response to different pathogenic factors (bacterial lippopolysacharides, proinflammatory cytokins). An excessive production of iNOS-derived NO leads to cytotoxicity. The limiting factor for NO synthesis is bioavailability of intracellular L-arginine (4). It is known that reciprocal regulation of arginase and NOS in L-arginine-metabolizing pathways exists. Arginase reciprocally regulates NOS expression by substrate depletion as it for their common substrate L-arginine with NOS (5). The proper balance between NOS (oxidative L-arginine methabolism) and arginase (non-oxidative L-arginine degradation) activity (expression) is essential for maintenance of NO homeostasis (6). The present study was, therefore, designed to evaluate the changes in the activity of L-arginine metabolic enzymes – NOS and arginase in spermatozoa of patients with infertility.

Materials and Methods

Subjects

This study involved 72 infertile men with different forms of pathospermia. Infertility is the inability of a sexually active, non-contracepting couple to achieve spontaneous pregnancy in one year. Patients were recruited between January 2014 and April 2016. A detailed medical history was performed for all studied cases. Exclusion criteria: subjects currently on any medication or antioxidant supplementation were not included. In addition, subjects with infertility over 10 years, azoospermia, genital infection, chronic illness and serious systemic diseases, smokers and alcoholic men were excluded from the study because of their well-known high seminal ROS levels and decreased antioxidant activity (7).

Ejaculates from a total of 72 infertile and 20 fertile healthy individuals were obtained. Subjects were classified into 4 groups as having different forms of pathospermia. According to semen analysis, oligozoospermia (the concentration of spermatozoa – $(11.95\pm2.35)\times10^6$ mL^{-1}; relative number of motile sperm – (42.33±4.95%) was found in 12 patients (16.7%), asthenozoospermia (the concentration of spermatozoa – $(44.30\pm5.35)\times10^6$ mL^{-1}; relative number of motile sperm – (24.05±5.35%) was detected in 17 patients (23.6%), oligoasthenozoospermia (the concentration of spermatozoa – $(9.95\pm1.65)\times10^6$ mL^{-1}; relative number of motile sperm – (26.05±4.25%) was observed in 10 patients (13.9%). Thirty-nine (54.2%) infertile men had leukocytes content in the semen lower than 1.0×10^6 mL^{-1}, only in 33 patients (45.8%) leukocytospermia was noted (the leukocytes content ranged from 1.0×10^6 mL^{-1} to 3.0×10^6 mL^{-1}; the concentration of spermatozoa – $(46.40\pm6.20)\times10^6$ mL^{-1}; relative number of motile sperm – (42.34±3.24%) which indicates inflammation in this group of men.

Semen samples of fertile men represent the control group which consisted of 20 healthy men with somatic fertility, normozoospermia (the concentration of spermatozoa – $(50.0\pm6.40\times10^6$ mL^{-1}; relative number of motile sperm – (52.86±3.22%) and confirmed parenthood (married for 3–10 years and have healthy 1–3 children). Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days' abstinence from sexual activity. After liquefaction at 37 °C with 5% CO_2 in air, semen samples were examined for volume, sperm concentration, pH, morphology and motility according to the World Health Organization guidelines (8).

Ethical approval

Before turning to study, all men were aware of patient information leaflets and gave informed consent to participate in research. Terms of sample selection meet the requirements of the principles of Helsinki Declaration on protection of human rights, Convention of Europe Council on human rights and biomedicine and the provisions of laws of Ukraine. Approval for study was taken from the ethics committe of Danylo Halytsky Lviv National Medical University. All patients and healthy donors gave written informed consent to participate in research (Ethical Committee Approval, protocol No 2 from February 16, 2015).

Cell preparation

Sperm cells were washed from semen plasma by 3 times centrifugation at 3000 × g for 10 min in

media which contained (mmol/L): 120 NaCl, 30 KCl, 30 Hepes (pH 7.4). The content of total protein in the samples was determined by Lowry method (9) using a kit to determine its concentration (»Simko Ltd«). The protein content did not exceed 50–75 mg/mL. Determination of ATPases activities was carried out in permeabilized spermatozoa. The detergent saponin in a final concentration of 0.5% was added to sperm suspension for permeabilization of sperm membranes. In permeabilized sperm cells the following activities were measured: calcium-dependent (cNOS, which is the sum of endothelial eNOS and neuronal nNOS), calcium-independent (iNOS) and arginase activity.

Arginase activity assay

Spermatozoa arginase activity was measured by determining levels of urea production. Briefly, incubation media of the following composition (mmol/mL): L-arginine – 100, $MnCl_2$ – 2, Tris-HCl – 20 (pH 9.5) was used. The protein concentration usually did not exceed 50–100 mg. The mixture was incubated at 37 °C for 90 min, and the reaction was stopped by adding 1 mL 50% trichloroacetic acid. After centrifugation, the urea was determined in the supernatant spectrophotometrically by measuring absorbance at 520 nm according to the assay kit »Simko Ltd«. Arginase activity was expressed as nmol urea per min per mg protein.

NOS activity assay

Spermatozoa NOS activity assay was performed by monitoring the rate of conversion of L-arginine into citrulline. Briefly, the samples aliquots that contained 300 µg protein were used to determine the total NOS activity. They were incubated for 60 min at 37 °C in a total volume of 1 mL substrate mixture (pH 7.0) of the following composition (mmol/mL): KH_2PO_4 – 50, $MgCl_2$ – 1, $CaCl_2$ – 2, NADPH (»Sigma«, USA) – 1, L-arginine – 2. The reaction was stopped by adding 0.3 mL of 2N $HClO_4$. As a control samples that contained the full substrate mixture previously denatured by 2N $NClO_4$ were used. The mixture was centrifuged at 3500 g for 10 min and the non-protein supernatant mixtures were used to test L-citrulline by highly specific method for color reaction with antipyrine. Its sensitivity is 0.2 mg of L-citrulline in 1 mL, so it can be used to study the NOS activity. Protein-free aliquot samples were mixed with 2 mL of reagent (1 mL of 59 mmol/L diacetyl monoxime (»Sigma«, USA) + 1 mL of 32 mmol/L antipyrine (»Sigma«, USA) + 55 µmol/L Ferrous (II) sulphate in 6N H_2SO_4) and boiled for 15 min in a water bath. After cooling the value of extinction was determined at 456 nm. The citrulline content was determined using a calibration graph. The method similar to the previous one was used to determine iNOS, but 2 µmol/L EDTA was added to the incubation mixture instead of $CaCl_2$. cNOS activity in the sperm cells was calculated as the difference between total NOS activity and iNOS activity. NOS activity was expressed as pmol citrulline/min per 1 mg of protein.

Statistical analyses

The data on the figures are expressed as means ± standard deviation. One-way ANOVA was performed to detect statistical significance. Differences with p-value < 0.05 were considered as significant.

Results

The enzymatic assay revealed significant difference in NOS and arginase activity in spermatozoa between patients with pathospermia and the control group (*Figure 1*).

It was found that total NOS activity in patients with oligozoospermia was 1.9-fold ($p<0.001$) greater than that in normozoospermic fertile men. In patients with asthenozoospermia and oligoasthenozoospermia the total NOS activities were 2.3-fold ($p<0.001$) greater than control group. The most expressed changes in total NOS activities were observed in patients with leukocytospermia – 3.4 fold ($p<0.001$) greater than that in normozoospermic men.

There was a significant decrease in arginase activity in sperm cells of patients with pathospermia compared the control group. We found that arginase activity in the sperm cells of men with oligozoo-, asthenozoo-, oligoasthenozoo- and leukocytospermia

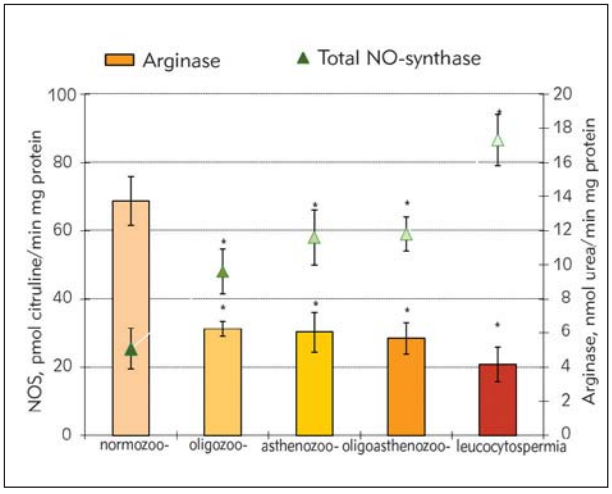

Figure 1 The NO-synthase and arginase activity in sperm cells of patients with different fertility potential.

Asterisks indicate significant differences relative to the control group (normozoospermia) at *$P<0.001$. In this and all other figures a columns show the mean and its standard deviation.

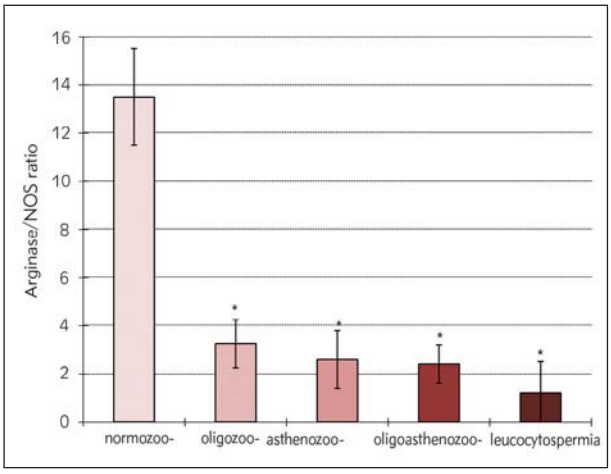

Figure 2 The arginase/NOS ratio in patients with different fertility potential.

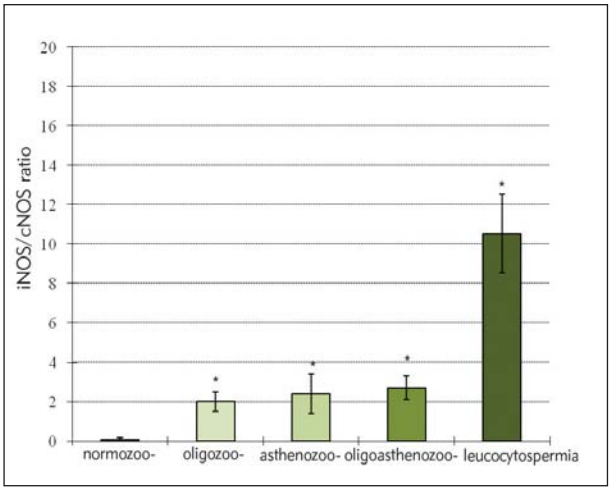

Figure 3 The iNOS/cNOS ratio in patients with different fertility potential.

was decreased in 2.1, 2.3, 2.4 and 3.3 folds ($p<0.001$) respectively.

The current investigation revealed that arginase/NOS ratio was significantly decreased in patients with pathospermia (*Figure 2*).

It is of interest to note that the arginase/NOS ratio was decreased over an order of magnitude in leukocytospermic patients relative to healthy controls. Thus, the decreased arginase/NOS ratio in pathospermic patients is largely due to the reduction of arginase activity and drastic activation of NOS, supporting their prominent role in the aetiology and/or pathophysiology of pathospermia.

It was found a reduced eNOS activity in patients with pathospermia relative to normozoospermic men, with no group difference in eNOS activity. At the same time sperm iNOS activity was increased significantly ($p<0.001$) in the all group of patients with pathospermia as compared with the control group. Men with leukocytospermia were distinguished to have the most expressed iNOS activity. The iNOS/cNOS ratio (Figure 3) was increased in patients with oligozoo-, asthenozoo-, oligoasthenozoospermia (2 – 2.7) and leukocytospermia (10.5) compared to normozoospermic men (0.06). Drastically increased iNOS/cNOS ratio in patients with decreased fertility potential indicating predominance of iNOS.

Discussion

In the present study, we found that NOS in the sperm cells of fertile men was characterized by the dominance of eNOS activity. This can be explained by the lack of factors that activate iNOS in healthy men with preserved fertility. In our study the iNOS activity detected at extremely low levels in sperm cells of healthy men compared to its constitutive isoform. It is not expressed constitutively by most cells, but its expression (activity) is induced by bacterial endotoxins, lipopolysaccharide and/or proinflammatory cytokines etc. However, it was confirmed participation of iNOS in both physiological (»basal«) synthesis of NO and in response to pathological stimuli (10, 11). It is believed that synthesis of »basal« NO is realized by cNOS, whereas iNOS provides additional amounts of NO in the cell under various pathological conditions. cNOS produces NO at low, physiologically appropriate concentrations, whereas iNOS produces NO in extremely high concentrations (12). Only low concentrations of NO are normally produced by cNOS in most cells physiologically, which play an important role in variety of physiological processes. On the other hand iNOS leads to relatively high concentrations of NO, from which nitrooxyl (NO^-) and peroxynitrite ($ONOO^-$) are being formed. Therefore, iNOS-derived NO is involved in oxidative stress.

Increased iNOS activity in infertile men indicates overproduction of NO in sperm cells of pathospermic patients. These results are in agreement with the finding of Zhao Y et al. (13) who reported increased NOS activity in sperm of infertile men. Likewise, Hadwan MH et al. (14) also showed that NOS activities in sperm cells and seminal plasma of men with asthenozoospermia were higher than men with preserved fertility. NO in concentrations above physiological norm can have genotoxic effects, damaging chemical structure of sperm DNA. It was shown that NO overproduction affect the formation and maturation of sperm cells, their mobility and morphology (15). It was found an inverse correlation between spermatograms parameters and NO concentration in men with normo-, astheno-, terato-, leukocyto- and leukoasthenospermia. Therefore, it was suggested that disruption of the sperm functions is associated with cytotoxic effects of excessive NO production (16).

Although NO is relatively unreactive molecule toward most biomolecules, it can interact with other free radical extremely rapidly which leads to increased formation of highly damaging reactive oxygen species peroxynitrite ($ONOO^-$). It is known that peroxynitrite can reduce level tetrahydrobiopterin, a co-factor of NOS. This leads to uncoupling of NOS, a state in which enzyme switch from NO production to superoxide anion generation causing further increases of $ONOO^-$ level (17).

Since arginase and NOS metabolize the same substrate therefore arginase should have a protective role with regard to increased iNOS activity (18). This regulatory mechanism is realized by reducing L-arginine availability for NOS to produce NO. It is known that in cells where both arginase and NOS are expressed, there is a reciprocal regulation between, meaning that factors which induce arginase expression (activity) can down-regulate the expression (activity) of NOS and vice versa. The concomitant expression of both iNOS and arginase can markedly reduce the nitric oxide overproduction in toxic levels in cells because of a decrease in the intracellular arginine content (19). However in our study, decreased arginase activity most likely led to increase NO production in sperm cells. Depressed arginase activity might be related to the enzyme inhibition by nitrite, the major stable metabolite of NO. It was shown a negative correlation between NO concentration and arginase activity in seminal plasma (20). In addition, N_G–hydroxyl-L-arginine (NOHA) generated by NOS is inhibitor of arginase. Increased concentrations of NOHA might increase L-arginine bioavailability for NO synthesis by reducing its bioavailability for arginase. It was shown that arginase inhibition would increase the L-arginine bioavailability to NOS and may reduce superoxide formation in diabetes by reversing eNOS uncoupling (21).

There is controversy concerning the ability of arginase and NOS to compete for the same substrate (L-arginine). NOS is the predominant pathway of L-arginine under physiological norm as NOS has a more than 1000-fold greater affinity to substrate than arginase (1 mmol/L for arginase and 1–10 μmol/L for NOS). Taking into account their significantly different values of affinity constant it is appear that they cannot compete. However, kinetic parameters of enzymes were determined in a closed system with isolated enzymes and do not take into account enzyme coupling, non-freely diffusible substrate pools, intracellular localization of the enzymes and substrate transporter expression and activity, diffusion gradients, and potential sequestration (22–24). It also should be taken into account that bioavailability of L-arginine is regulated by its *de novo* biosynthesis and activity of transporting systems of L-arginine.

The finding in the present study of decreased arginase activity is in agreement with earlier reports showing that enzyme activity was significantly higher in the fertile group than the infertile patients (14). Also there was a positive correlation between the sperm concentration and sperm motility with arginase activity (20). However, these results are in disagreement with other older studies which showed that arginase activity in sperm cells was greater in infertile men with oligozoospermia than in healthy men (25).

The most expressed changes in NO-synthase and arginase activity were observed in patients with leukocytospermia. It is known that leucocytes negatively affect sperm cells, stimulate the formation of reactive oxygen species, induction and development of oxidative stress (26). This leads to the disruption of L-arginine metabolism. We suggest that a decrease in the cellular energy substrates may be responsible for altered L-arginine metabolism in patients with asthenozoo- or/and oligoasthenozoospermia and disturbances of spermatogenesis can lead to altered L-arginine metabolism in patients with oligozoospermia.

The changes in arginase activity may subsequently alter L-arginine availability for NOS and thus influence NO production. In a situation of decreased arginase activity, more arginine may be available for NOS which results in increased NO production. In patients with decreased fertility potential the NO overproduction by iNOS may be contributing to the suppression of the arginase system, causing further disturbances in L-arginine metabolism.

To the best of our knowledge, the present study describes the peculiarities of the L-arginine metabolism, taking into account its parallel NOS and arginase pathways in sperm cells of infertile men with different forms of pathospermia compared with their age-matched fertile control cases. The present study provides further evidence of altered sperm arginine metabolism in infertile men, which enhances our understanding of the pathogenesis of male infertility. Therefore, determination of the dynamics of the activity of NOS isoforms may be an additional prognostic criteria/marker used for confirmation of infertility and for the evaluation of effectiveness of treatment. Further research into the mechanism causing NO overproduction by iNOS and resultant arginase inhibition may lead to promising novel therapeutic strategy in the future. The changes of L-arginine metabolism in spermatozoa merit future research of kinetic properties of arginase and NO-synthase.

Conclusion

These observations provide evidence for a disturbed balance between the L-arginine metabolic pathways in sperm cells of infertile men. This imbalance includes the considerable activation of the inducible isoform of NO-synthase accompanied by significant inhibition of its constitutive isoform which

indicates disturbances in NO production. In patients with decreased fertility potential the arginase/NOS was shifted towards predominance of iNOS-derived NO production.

Study limitation

There are some limitations in the present study. First, our control group (normozoospermic men with proven fertility) and pathospermic patients contained a highly heterogeneous population, with large variations in spermogram parameters and infertility histories. Second, the present study investigated how sperm L-arginine metabolism was affected in pathospermic patients only with 72 cases. It is therefore essential to validate our findings with greater sample sizes and to determine the disease specificity (secretory or excretory infertility, varicocele or others) by comparing spermogram parameters. Nevertheless, the present study extends previous work and provides further evidence of altered L-arginine metabolism in sperm cells in pathospermia.

Funding. The publication contains the results of studies conducted by President's of Ukraine grant for competitive projects (project No 63/97-2016 from 10.08.2016 »Molecular biological regulatory mechanisms of disturbance of fertilizing ability spermatozoa and the development of new immuno-biochemical diagnostic methods of fertility in men« (scientific supervisor – Doc. Sci. D.Vorobets) of the State Fund for Fundamental Research.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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Received: May 24, 2017

Accepted: September 14, 2017